North Wind

[redacted]

Poultry Quantitative PCR Analytical Summary

09-17-07

Overview:

The objective of this project was to quantify the number of poultry specific Brevibacteria biomarker gene copies contained in water, soil, and/or litter samples using quantitative polymerase chain reaction (qPCR). The client is Camp, Dresser and McKee. Table 1 describes the sample matrix and the condition of the samples upon arrival to the analytical laboratory.

Table 1. Description of samples and volume or mass filtered for DNA extraction.

Sample ID	Matrix–Date Sampled	Condition Received/Observations	Volume Filtered (L) or Mass Extracted (g)
EOF-spr-010-5-9-06	Water–5/9/06	Cold/bottle intact	40 mL
EOF-spr-17A-01-5-1-06	Water-5/1/06	Cold/bottle intact	30 mL
EOF-spr-023-6-18-06	Water-6/18/06	Cold/bottle intact	25 mL
LAL16-SPR2-7-18-06	Water-7/18/06	Cold/bottle intact	100 mL
LAL16C-2-7-18-06	Soil-7/18/06	Cold/sealed bag	0.35 g
LAL11C-2-6-28-06	Soil-6/28/06	Cold/sealed bag	0.57 g
HFS16-BF1-01-6-15-06	Water-6/15/06	Cold/bottle intact	400 mL
SALspr-6-28-06	Water-6/28/06	Cold/bottle intact	150 mL
LAL15-SP2-7-11-06	Water-7/11/06	Cold/bottle intact	250 mL
RS-PRICEcrk-01-4-29-06	Water-4/29/06	Cold/bottle intact	150 mL
RS-574-BIO	Water	Cold/bottle intact	200 mL
Lk04-0-01-5-16-06	Water-5/16/06	Cold/bottle intact	250 mL
HFS28A-BF1-01-6-15-06	Water-6/15/06	Cold/bottle intact	400 mL
Rs-1-01-8-8-06	Water-8/8/06	Cold/bottle intact	500 mL
FAC-01A-1	Litter–2/2/06	Cold/sealed bag	0.25 g
FAC-01A-2	Litter–2/2/06	Cold/sealed bag	0.25 g
FAC-01A-3	Litter–2/2/06	Cold/sealed bag	0.25 g
FAC-01A-4	Litter–2/2/06	Cold/sealed bag	0.25 g
FAC-01A-5	Litter–2/2/06	Cold/sealed bag	0.25 g
FAC-01B-1	Litter–2/2/06	Cold/sealed bag	0.25 g
FAC-01B-2	Litter–2/2/06	Cold/sealed bag	0.25 g
FAC-01B-3	Litter–2/2/06	Cold/sealed bag	0.25 g
FAC-01B-4	Litter–2/2/06	Cold/sealed bag	0.25 g
LAL8-A-2-6-19-06	Soil–6/19/06	Cold/sealed bag	0.25 g
LAL16B-2-7-18-06	Soil–7/18/06	Cold/ sealed bag	0.25 g
RS-901-BIO	Water–8/9/06	Cold/ bottle intact	250 mL
LAL16-GW2-7-18-06	Water–7/18/06	Cold/bottle intact	250 mL
CollinsWell#1-7-7-06	Water–7/7/06	Cold/bottle intact	250 mL
66783-7-26-06	Water–7/26/06	Cold/bottle intact	300 mL
LK-01-0-01-8-9-06	Water–8/9/06	Cold/bottle intact	300 mL
Hester-498-8-10-06	Water–8/10/06	Cold/bottle intact	250 mL

[redacted] STOK0029428

Exhibit 6

North Wind

The samples arrived in good condition at 4 deg C. All samples were received within 24 hours of sample collection. Upon arrival, the samples were filtered and frozen for storage at -80 deg C until the DNA extraction was performed. Following DNA extraction, the samples were first subjected to polymerase chain reaction (PCR) using universal bacterial probes in order to verify amplifiable DNA was present in the sample. In addition, for the 16S rRNA gene, a "nested" qPCR approach can be applied in which the universal bacterial PCR-amplified DNA is used as the template in a qPCR reaction. Although the results from the nested qPCR cannot be quantified per se, they can be used to lower the detect limit for the qPCR in order to determine if the poultry specific Brevibacteria biomarker gene is present at concentrations lower than the method detect limit (MDL) using the groundwater DNA extractions. The results of these studies are described here.

Methods:

DNA Extraction. For soil and/or litter samples, DNA was extracted from 0.25 g of soil or litter using the FastDNA®SPIN® Kit for Soil protocol. For surface water shipped to the laboratory, between 100 and 1,000 mL of groundwater was filtered through a Supor-200, 0.2 µm filter. The filters were frozen at -80 deg C and then shattered. Next, each sample tube was amended with 2 mL of DNA-free water, vortexed vigorously for 15 minutes, and the liquid volume was partitioned into DNA extraction tubes. DNA extractions were performed using the FastDNA®SPIN® Kit for soil according to the manufacturer's instructions. All DNA extractions were cleaned using an ethanol precipitation method. Community DNA was eluted in nuclease-free water (50 uL) and stored at -20 deg C.

Amplification of Bacteria. The PCR was used to amplify nearly full-length 16S rDNA genes from *Bacteria*. Each 25-µL PCR reaction included 1 X PCR buffer , 1.5 mM $MgCl_2$, 0.5 uM each 8F forward and 907R reverse primer, 1 u/50uL Taq DNA polymerase, 0.2 mM dNTP, 1 uL template DNA, and 20 uL molecular-grade water. Amplification was performed on a MJ Research Peltier Gradient thermocycler using the following regime: 94 deg C (5 min) followed by 30 cycles of 94 deg C (1 min), 53.5 deg C (1 min), and 72 deg C (1 min 50 sec). The reaction was finished with an additional 7 minutes at 72 deg C. PCR products were examined by UV light in a 1% agarose gel stained with ethidium bromide to confirm specificity of the amplification reactions.

Sepharose cleanup. Any sample not amplifying in the PCR was processed through a Sepharose CL-4B (Sigma-Aldrich) size exclusion gel chromatography cleanup. Briefly the micro-bio spin columns (Bio-Rad) were packed with sterile Sepharose CL-4B and washed with Tris-HCl buffer (pH 8). Sample was added to the packed gel column and eluted by spinning in a micro-centrifuge.

Detection of a Poultry Specific Brevibacteria Biomarker. The qPCR methods for assessing the 16S rRNA gene are very sensitive in detecting specific DNA fragments. The detection limit for the methods used is approximately 6 gene copies per uL of the DNA extraction. Biomarker DNA was cloned into a plasmid was used as the source of the quantitative standards used in the analysis. Plasmid DNA containing the target 16S rRNA gene from the poultry specific Brevibacteria biomarker was purified and quantified fluorometrically. Based on the known size of the plasmid and insert, DNA concentrations were converted to insert copy numbers. A dilution series spanning seven orders of magnitude was generated using known concentrations of each plasmid. Amplification and detection of the DNA was performed using the MJ Chromo-4 System. The acceptance criterion for the standard curve is a linear R^2 value of greater than 0.995.

To determine qPCR results, sample DNA diluted to a final concentration of 15 ng/5uL DNA was combined with following reagents to reach a final concentration of 1 X SYBR Green Master Mix, 0.5 uM 157F and 727R primer and water to reach 20 uL and 5 uL of diluted sample DNA. Amplification was performed on the MJ Research PTC-2004 thermocycler using the following regime: 50 deg C (2 min), 95 deg C (15 min), 40 cycles of 95 deg C (30 sec), 60 deg C (1 min), plate read and 50 deg C (5 min). The melting curve was determined using the following protocol: heat from 60 deg C to 90 deg C by 0.3 deg C increments,

North Wind

and holding for 5 seconds before reading the fluorescence of the samples.

Nested qPCR results were determined by purifying the PCR products using the QIAquick PCR Purification Kit, as per the manufacturer's protocol, and then running the purified samples through qPCR, as described above.

QA/QC Requirements. To determine if and where potential contamination or interference occurred during sample processing, positives and reagent blanks or negatives and matrix spikes of the PCR and qPCR samples were prepared. A positive control consisting of pure DNA (known to amplify by specific DNA primers) was used for the PCR and qPCR procedure. A matrix spike consisting of pure DNA (known to amplify by specific DNA primers) was used for the PCR and qPCR procedure. Negative controls consisted of water-only blanks for the PCR and qPCR procedure. The qPCR reactions were run in triplicate for each sample to determine the reproducibility of the method.

Results:

The samples arrived at the lab in good condition at 4 deg C with ice still in the cooler. The samples were filtered in the lab, and the filters were immediately placed in a -80 deg C freezer and stored until the DNA extraction was performed. Table 2 summarizes the qPCR analysis of the poultry project samples. The DNA extraction negative control and all PCR negative controls did not amplify any product. In addition, all calibration control checks were within acceptable values.

Table 2. Results of molecular analyses for the poultry samples.

Sample ID	Matrix	DNA (ng/L or ng/g)	qPCR Poultry Specific Biomarker (copies/ul water or g soil or g litter)			qPCR matrix spike amplified? *	Nested qPCR amplified? *	Biomarker melt peak identified?	Other melt peaks observed?
EOF-spr-010-5-9-06	Water	1.7	1.05E+07	±	1.70E+06	Yes	N/A	Yes	No
EOF-spr-17A-01-5-1-06	Water	72.5	2.48E+06	±	4.71E+05	Yes	N/A	Yes	Yes
EOF-spr-023-6-18-06	Water	4.3	1.11E+05	±	2.49E+03	Yes	N/A	Yes	No
LAL16-SPR2-7-18-06	Water	-1.0	0.0			Yes	No	N/A	N/A
LAL16C-2-7-18-06	Soil	14.5	0.0			No, Inhibited	N/A, Inhibited	N/A	N/A
LAL11C-2-6-28-06	Soil	73.2	0.0			Yes	Yes	Yes	No
HFS16-BF1-01-6-15-06	Water	6.8	4.00E+03	±	1.60E+03	Yes	N/A	Yes	No
SALspr-6-28-06	Water	-0.6	5.82E+02	±	1.56E+02	Yes	N/A	Yes	No
LAL15-SP2-7-11-06	Water	5.0	2.89E+03	±	7.69E+02	Yes	N/A	Yes	No
RS-PRICEcrk-01-4-29-06	Water	4.7	3.45E+05	±	1.43E+05	Yes	N/A	Yes	No
RS-574-BIO	Water	6.7	1.80E+05	±	6.09E+04	Yes	N/A	Yes	No
Lk04-0-01-5-16-06	Water	6.8	3.69E+03	±	3.24E+03	Yes	N/A	Yes	No
HFS28A-BF1-01-6-15-06	Water	-0.7	2.48E+03	±	1.28E+03	Yes	N/A	Yes	Yes

Rs-1-01-8-8-06	Water	7.0	3.19E+04	±	6.75E+03	Yes	N/A	Yes	Yes
FAC-01A-1	Litter	33.7	2.18E+09	±	3.53E+08	Yes	N/A	Yes	No
FAC-01A-2	Litter	4.7	2.47E+08	±	3.22E+07	Yes	N/A	Yes	No
FAC-01A-3	Litter	-0.5	2.67E+07	±	2.69E+06	Yes	N/A	Yes	No
FAC-01A-4	Litter	3.4	1.49E+08	±	1.10E+07	Yes	N/A	Yes	No
FAC-01A-5	Litter	4.1	5.67E+08	±	3.75E+07	Yes	N/A	Yes	No
FAC-01B-1	Litter	94.5	3.94E+09	±	6.28E+08	Yes	N/A	Yes	No
FAC-01B-2	Litter	40.5	2.66E+09	±	7.57E+08	Yes	N/A	Yes	No
FAC-01B-3	Litter	34.5	4.75E+06	±	4.23E+06	Yes	N/A	Yes	No
FAC-01B-4	Litter	117.1	5.99E+09	±	1.74E+09	Yes	N/A	Yes	No
LAL8-A-2-6-19-06	Soil	22.34	7.00E+03	±	4.43E+02	Yes	N/A	Yes	No
LAL16B-2-7-18-06	Soil	28.94	2.91E+05	±	1.95E+04	Yes	N/A	Yes	No
RS-901-BIO	Water	1.3	0.0			Yes	No	N/A	N/A
LAL16-GW2-7-18-06	Water	2.0	0.0			Yes	No	N/A	N/A
CollinsWell#1-7-7-06	Water	4.0	0.0			Yes	No	N/A	N/A
66783-7-26-06	Water	0.8	0.0			Yes	No	N/A	N/A
LK-01-0-01-8-9-06	Water	5.2	0.0			Yes	No	N/A	N/A
Hester-498-8-10-06	Water	2.9	0.0			Yes	No	N/A	N/A

N/A, not applicable. The sample was not run with the nested qPCR assay and/or the biomarker melt peak was not identified because none was detected in the qPCR sample run.

* inhibited indicates that the sample did not amplify with qPCR even after a sepharose cleanup was performed and the sample was diluted to a lower DNA concentration.